STUDIES ON THE IMMUNOCHEMICAL TECHNIQUE FOR DETECTION OF SELECTED FUNGAL AND DINOFLAGELLATE TOXINS

Second annual progress report for Contract

DAMD17-82-C-2021

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During the second year (August 1, 1982 to July 31, 1983), conditions for improving antibody production against T-2 toxin were studied. T-2 HS-BSA which has 10-15 moles of T-2 per mole of BSA was found to be a better immunogen than T-2-HG-BSA. Antibody titers as high as 11,000-14,000 were obtained from rabbits after repeated booster injections. High specific radioactive T-2, DAS, DOVE were prepared. With combination of the high specific radioactive T-2 toxin and high titer antibody, as little as 25 pg of T-2 toxin can be detected by the RIA in each assay. An indirect ELISA which permits detection of 0.2-1 ppb of T-2 toxin in urine, serum and milk was also established. Antibody against T-2 toxin has been used to localize T-2 toxin in different tissues and organs of mice by an immunohistochemical technique developed in our laboratory. A collaborative study with Dr. Hunter revealed that antibody titers of T-2 toxin increased

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drastically in an immunomodulation system in mice. Titers as high as 50,000 were obtained in mice 10 days after immunization with T-2-HS-IgG (goat). Antibodies against diacetoxyscirpenol (DAS), and deoxyverrucarol (DOVE) were obtained from rabbits after immunizing with DAS-hemisuccinate (HS)-BSA, DAS-hemiglutarate (HG)-BSA and DOVE-HS-BSA. DAS-HG-BSA was found to be a better immunogen than DAS-HS-BSA for the production of antibody against DAS. The antibody for DAS is most specific for DAS. However, DOVE antibodies were found to be less specific. Radioimmunoassays for both DAS and DOVE were established, and the detection limits were found to be 0.5 ng/assay and 0.25 ng/assay for DAS and DOVE, respectively. A number of approaches have been used for the preparation of vomitoxin (VT)-BSA conjugates which were subsequently used in the immunization for the production of antibody against VT. The antibodies elicited by rabbits appeared to be not very specific for VT. Antibodies against saxitoxin (STX) were obtained after immunizing with an STX-BSA which was prepared by cross-linking with formaldehyde. An indirect ELISA for STX which can detect as little as 25 pg of STX was established. The STX antibodies were capable of neutralizing the toxic effect of STX by injecting it into mice one day before challenging with STX. During the present contract period, a total of 80 mg of T-2 BSA conjugate with 10-15 moles of T-2 toxin per mole of BSA, 5 mg T-2 enzymes, 22 mg of T-2 polylysine, 57 ml of antisera against T-2 toxin, 50 mCi of ^{3}H-T-2 toxin, 6 mg of DOVE-HS-BSA, 2 mg of DOVE-HS-peroxidase, 2.5 mg DOVE-HS-polylysine and 5 ml of antiserum against STX were prepared and delivered. In addition, 10 mg of T-2-HS-IgG (goat) and 13 mg of DOVE-HS-IgG (goat) were prepared and delivered to Dr. Hunter.

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STUDIES ON THE IMMUNOCHEMICAL TECHNIQUES FOR DETECTION OF SELECTED FUNGAL AND DINOFLAGELLATE TOXINS

Annual Report

F. S. Chu

August 15, 1983

Supported by

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University of Wisconsin-Madison
Madison, Wisconsin 53706

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SUMMARY

During the second year (August 1, 1982 to July 31, 1983), conditions for improving antibody production against T-2 toxin were studied. T-2 HS-BSA which has 10-15 moles of T-2 per mole of BSA was found to be a better immunogen than T-2-HG-BSA. Antibody titers as high as 11,000-14,000 were obtained from rabbits after repeated booster injections. High specific radioactive T-2, DAS, DOVE were prepared. With combination of the high specific radioactive T-2 toxin and high titer antibody, as little as 25 pg of T-2 toxin can be detected by the RIA in each assay. An indirect ELISA which permits detection of 0.2-1 ppb of T-2 toxin in urine, serum and milk was also established. Antibody against T-2 toxin has been used to localize T-2 toxin in different tissues and organs of mice by an immunohistochemical technique developed in our laboratory. A collaborative study with Dr. Hunter revealed that antibody titers of T-2 toxin increased drastically in an immunomodulation system in mice. Titers as high as 50,000 were obtained in mice 10 days after immunization with T-2-HS-IgG (goat). Antibodies against diacetoxyscirpenol (DAS), and deoxyverrucarol (DOVE) were obtained from rabbits after immunizing with DAS-hemisuccinate (HS)-BSA, DAS-hemiglutarate (HG)-BSA and DOVE-HS-BSA. DAS-HG-BSA was found to be a better immunogen than DAS-HS-BSA for the production of antibody against DAS. The antibody for DAS is most specific for DAS. However, DOVE antibodies were found to be less specific. Radioimmunoassays for both DAS and DOVE were established, and the detection limits were found to be 0.5 ng/assay and 0.25 ng/assay for DAS and DOVE, respectively. A number of approaches have been used for the preparation of vomitoxin (VT)-BSA conjugates which were subsequently used in the immunization for the production of antibody against VT. The antibodies elicited by rabbits appeared to be not very specific for VT. Antibodies against saxitoxin (STX) were obtained after immunizing with an STX-BSA which was prepared by cross-linking with formaldehyde. An indirect ELISA for STX which can detect as little as 25 pg of STX was established. The STX antibodies were capable of neutralizing the toxic effect of STX by injecting it into mice one day before challenging with STX. During the present contract period, a total of 80 mg of T-2 BSA conjugate with 10-15 moles of T-2 toxin per mole of BSA, 5 mg T-2 enzymes, 22 mg of T-2 polylysine, 57 ml of antisera against T-2 toxin, 50 mCi of ^{3}H-T-2 toxin, 6 mg of DOVE-HS-BSA, 2 mg of DOVE-HS-peroxidase, 2.5 mg DOVE-HS-polylysine and 5 ml of antiserum against STX were prepared and delivered. In addition, 10 mg of T-2-HS-IgG (goat) and 13 mg of DOVE-HS-IgG (goat) were prepared and delivered to Dr. Hunter.

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FOREWORD

The following is the second annual report (12 months) of the work performed under contract No. DAMD17-82-C-2021 during the period of August 1, 1982 to July 31, 1983. The work was carried out at the Food Research Institute, University of Wisconsin-Madison under the direction of the principal investigator, Dr. F. S. Chu and co-principal investigator, Dr. E. J. Schantz. The contract officer is Dr. Robert W. Wannemacher, Jr. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. INTRODUCTION

The goal of this contract is to develop a method for the production of antibodies against several selected mycotoxins and dinoflagellate phytotoxins and subsequently to develop a radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA) for toxin determination as well as to use these antibodies as prophylactic agents. To achieve the main objective, the following specific tasks were planned:

(a) development of methods for conjugation of STX and its related dinoflagellate toxins to protein carrier;

(b) development of methods for conjugation of VT and other related trichothecene mycotoxins to protein carrier;

(c) elicit antibodies against the toxin-protein conjugates;

(d) development and refinement of RIA and enzyme-linked immunosorbent assay (ELISA) for mycotoxins and their application for analysis of these toxins in military foods;

(e) investigation of the in vitro and in vivo neutralization of mycotoxins and STX by antibody;

(f) immunohistochemical studies on T-2 toxin;

(g) attempts to elicit monoclonal antibody against vomitoxin and diacetoxyscirpenol;

and (h) prepare and deliver hapten, antibody and enzyme-linked toxin to the US Army Medical Research Institute of Infectious Diseases (USAMRIID).

During the first nine months (Nov. 1, 1981 to July 31, 1982), effective methods for the preparation of hemisuccinate (HS) and hemiglutarate (HG) of T-2 toxin, HS of diacetoxyscirpenol (DAS), and O-carboxymethyl oxime (O-CM) of vomitoxin (deoxynivalenol, VT) were developed. Methods for the preparation of reduced saxitoxin (STX) as well as HS of reduced saxitoxin (STXOL) were also established. These derivatives were chemically characterized and were conjugated to bovine serum albumin (BSA) for subsequent immunization. They also have been conjugated to horseradish peroxidase (HRP) for direct enzyme linked immunosorbent assay (ELISA) and to polylysine and to hemocyanin for indirect ELISA. The toxicity of T-2 HS was determined in mice and was found to be less toxic than T-2 toxin. The LD_{50} for T-2 HS was estimated to be around 7.5 mg/kg as compared with T-2 toxin which had a LD_{50} around 3-4 mg/kg. For antibody production, a total of 26 rabbits were immunized with various toxin-BSA conjugates. The antibody titers were measured by either a RIA method using tritiated reduced VT or ^{3}H-T-2 toxin or tritiated STXOL, or by both direct and indirect ELISA methods using the toxin-HRP or goat antirabbit IgG-HRP

as the enzyme marker. Antibody titers varied considerably with the conjugates used but none was found to be very immunogenic. Whereas antibody titers against STX and VT were demonstrated by an indirect ELISA method for most immunized rabbits, the titers were very weak as determined by the direct ELISA. During the first nine months of the contract period, a total 143 mg of T-2 BSA conjugate with varied degrees of T-2 toxin per mole of BSA, 5 mg of T-2 enzymes, 4 mg of T-2 polylysine, 34 ml of antisera against T-2 toxin, 20 mg of T-2 hemocyanin, and 1 mCi of H3-T-2 toxin were prepared and delivered. Details of the first nine months' studies were summarized in our first annual report (August 15, 1982).

The progress of our second year's work (August 1, 1982 to July 31, 1983) is presented in this report.

II. WORK DONE DURING THE SECOND YEAR

A. Studies on T-2 toxin:

1. Improvement for the production of antibody in rabbits: A systematic study for the factors affecting the production of antibody against T-2 toxin in rabbits was carried out. Both T-2 hemisuccinate (T-2 HS) and T-2 hemiglutarate (T-2 HG) were used as the immunogens in the test. The results as summarized in Fig. 1 and 2 indicate that: (1) T-2 HS-BSA was a better immunogen than T-2 HG-BSA; however, high variation of the antibody titers was observed among the rabbits which were immunized with T-2 HS-BSA; (2) rabbits immunized with conjugates contained moderate amounts of T-2 toxin per mole of BSA, i.e., 10-15 moles of T-2 per mole of BSA had best antibody titers; (3) the optimal booster injection time was found to be around every 5-7 weeks; (4) highest antibody titers (11,000) were obtained from the rabbits 43 weeks after initial immunization and with 5 booster injections. With this kind of antibody titer, one ml of antiserum can run as much as 11,000 of RIA with good reliable results.

2. Improvement on the preparation of radioactive T-2 toxin: An improved method for preparation of highly specific radioactive T-2 toxin was established. The problem of degradation of highly specific tritiated T-2 toxin was overcome by storing the labelled toxin in ethanol at a concentration of less than 1 mCi per ml. Two radioactive T-2 epimers with specific radioactivity as high as 19.5 Ci/mmole were prepared and delivered. The effectiveness of use of these two epimers in the RIA of T-2 toxin was tested. Alpha epimer (natural form) was found to be more effective than the beta form in the RIA. Not only was much less antibody required, the sensitivity of the RIA also improved considerably. With the new ^{3}H-T-2 toxin, the sensitivity of RIA is comparable to that of ELISA. As little as 25 pg of T-2 toxin could be monitored by RIA.

3. Establishment of an indirect ELISA for analysis of T-2 toxin in serum and urine: Details of this study are presented in Appendix I.

4. Immunohistochemical studies on T-2 toxin: Details of this study are presented in Appendix II.

5. Production of antibody against T-2 toxin in Balb/c mice: As an initial approach for producing monoclonal antibody against T-2 toxin, the conditions for production of antibody in Balb/c mice were studied. Both the amount (2.5, 5 and 10 µg per mouse) and type of immunogens (T-2 HS-BSA, T-2 HG-BSA, and T-2-HS-hemocyanin) used in the immunization were investigated. A multiple site injection method (subcutaneous) was used in the initial immunization. All the immunogens were mixed with an equal volume of complete Freund's adjuvant before injection. Booster injections were made at the 9th and 14th weeks after initial injection. The immunogens were mixed with incomplete Freund adjuvant and injected in the mouse via the i.p. route for the booster injections. Highest antibody titers against T-2 toxin were obtained from mice 5 days after the second booster injection.

6. Collaborative studies with Dr. Hunter: During this year, we have conjugated T-2 HS to goat IgG, and T-2 to polylysine which were subsequently sent to Dr. Hunter for immunization in mice in an immunomodulation system. High antibody titers (50,000) were obtained when mice were injected with T-2-HS-IgG together with goat antimouse IgG. Details of this study will be documented by Dr. Hunter.

B. Studies on DAS:

1. Preparation of tritiated DAS: Highly specific tritiated DAS (19.5 Ci/mmole) was prepared using a procedure similar to that described for T-2 toxin. The labelled toxin was purified by semipreparative HPLC before use.

2. Production of antibody against DAS: Antibody against DAS was obtained from rabbits 6 weeks after immunizing with DAS-HS-BSA and DAS-HG-BSA (both were 12 moles of T-2/mole of BSA). Both ELISA and RIA were used for monitoring the antibody titers and the results for RIA are given in Fig. 3. It is apparent that DAS-HG-BSA was a better immunogen than DAS-HS-BSA for eliciting antibody against DAS in rabbits. A preliminary study on the specificity of the DAS antibody as tested by a competitive RIA was carried out. The antibody appears to be most specific for DAS, with little, if any, cross-reactivity toward T-2 and VT (Fig. 4). The lower limits for detection of DAS by the RIA were found to be around 0.5 ng per assay. We have used this antibody to monitor the production of DAS in culture medium which had been inoculated with various Fusarium spp. Antibody titers against DAS were also demonstrated by a direct ELISA in which DAS-HS was conjugated to peroxidase. However, because the DAS-HS peroxidase was not very stable, we still have problems in using ELISA for DAS analysis.

C. Studies on vomitoxin (VT):

1. Preparation of VT derivatives: During the contract period, a total of 6 different VT derivatives were prepared and subsequently conjugated to BSA, polylysine, and peroxidase by either water soluble carbodiimide method or mixed anhydride technique. These derivatives include carboxymethyl oxime (CMO) of VT, VT-HS, triacetyl(TA)-VT, TA-8-OH-VT, TA-8-HS-VT, TA-CMO-VT and thioglycolic-VT. CMO-VT was prepared by reaction of VT with carboxymethoxylamine•HCl in the presence of 5% NaOH. Hemisuccinate of VT (VT-HS) was prepared after reaction of succinic anhydride with 8-OH-VT which was prepared by reduction of VT with sodium borohydride. Triacetyl-VT was prepared by reacting VT with acetic anhydride in the presence of pyridine. After acetylation, it was reduced to TA-8-OH-VT with sodium borohydride and subsequently converted to TA-8-HS-VT by reaction with succinic anhydride. A TA-CMO-VT was prepared by acetylation of CMO-VT in the presence of pyridine. Thioglycolic-VT was prepared by reaction VT directly with thioglycolic acid.

2. Production of antibody against VT: All the above derivatives were conjugated to BSA, CMO, also conjugated to ovalbumin (OVA), and subsequently used for immunization. Antibody titers as measured by either direct ELISA or by an indirect ELISA were demonstrated in most rabbits immunized with the immunogens. A typical direct ELISA titration curve is shown in Fig. 5. Results for the cross-reactivity of different VT antibodies with peroxidase conjugated with different VT derivatives are summarized in Table I. The antibodies reacted most effectively with the VT-protein conjugate but not with free VT. Typical competitive direct and indirect ELISA displacement curves for VT are shown in Figs. 6 and 7. The minimum detection levels for VT by the direct and indirect ELISA were found to be 5 and 50 ng (or 0.1 and 1.0 µg/ml) per assay. Thus, the sensitivities are not adequate for VT analysis.

D. Studies on deoxyverrucarol (DOVE):

The objective of this study was to test if rabbits elicit antibodies which recognize most type A trichothecene mycotoxins after immunizing with a derivative of DOVE.

1. Preparation of DOVE-HS and radioactive DOVE: Hemisuccinate of DOVE was prepared according to methods similar to the preparation of T-2 HS and was conjugated to BSA via the water soluble carbodiimide method. Radioactive DOVE was prepared by oxidized DOVE with CrO_3(2 pyridine) in dry methylene chloride, with highly specific ^3H-$NaBH_4$ (78 Ci/mmole). The final products were purified by HPLC and analyzed by MS and TLC.

2. Production of antibody: Three rabbits were each immunized with 500 µg of DOVE-HS-BSA (15 M DOVE/M BSA). Antibody titers were measured by both indirect ELISA and by RIA. In the

indirect ELISA, DOVE-HS-polylysine was coated to the plate, followed by incubation with rabbit antisera and goat-antirabbit IgG-peroxidase conjugate. For RIA, a preliminary experiment was carried out to determine the affinity of the antiserum with tritiated DAS, DOVE and T-2 toxin. At the same antiserum concentration, binding of radioactivity to the antiserum was highest for the tritiated DAS among 3 ligands tested. Therefore, DAS was selected as a radioactive marker for subsequent antibody titer determination. Highest titer was obtained from one rabbit 5 weeks after initial immunization (Fig. 8). Subsequent ELISA studies also show that the antibody cross-reacts with DAS. However, in a competitive RIA in which unlabelled DOVE, T-2 and DAS were used to displace the binding of ^{3}H-DAS with the antibody, we found that DOVE was most effective in displacing the radioactive toxin. This result suggests that the antibodies have higher affinity toward DOVE than either DAS or T-2 toxin. Most recently, we have prepared very high specific activity of radioactive ^{3}H-DOVE and confirmed that this antibody has highest affinity to DOVE. The detection limit of RIA for DOVE was around 0.25 ng/assay.

E. Studies on saxitoxin (STX):

1. Preparation of different STX derivatives for antibody production: During the second year, we continued to boost the rabbits which had been immunized with STX-HS-BSA. In addition, a decarbamoyl-STX was prepared by hydrolysis of STX in the presence of 6N HCl, and was subsequently conjugated to BSA (DEC-STX-BSA) for immunization. Saxitoxin was also conjugated to BSA (STX-BSA) by cross-linking with formaldehyde. Whereas antibodies against STX were demonstrated after rabbits immunized with all the conjugates were tested, only those obtained from rabbits which had been immunized with STX-BSA were useful for ELISA.

2. Development of an indirect ELISA for STX: An indirect ELISA for detection of STX and STX antibody was developed. Antibodies against STX were demonstrated in rabbits by an indirect ELISA 5 weeks after they were immunized with 500 µg of STX-BSA which was prepared by cross-linking the toxin to BSA with formaldehyde. In the ELISA, STX-BSA conjugate was precoated onto the microtiter plate, followed by incubation with standard toxin and STX antibody. The amount of antibody bound to the solid phase was then determined by incubation with goat antirabbit-peroxidase conjugate and subsequently with peroxidase substrate. The lower limit for detection of STX by the indirect ELISA was around 25 pg per assay (Fig. 9).

3. Neutralization of toxicity of STX with antibody in mice: The ability of antibody to neutralize STX toxicity was tested. Strain CF-1 mice, 3 per group, were each injected with one ml of different dilutions of antiserum obtained from a rabbit 8 weeks after immunization with STX-BSA. One day after injection of antisera, the mice were each challenged with 0.35 µg of STX by an i.p. injection, and the time of death for each mouse was recorded. The results showed that there was a slight protection for the mice pretreated with

antiserum at a one to 10 dilution. Complete protection was observed for the mice preinjected with one ml of a one to 5 dilution of antiserum.

F. Preparation of deliverables:

During the present contract period, a total of 80 mg of T-2-HS-BSA conjugate with 10-15 moles of T-2 toxin per mole of BSA, 5 mg T-2 enzymes, 22 mg of T-2-HS polylysine, 57 ml of antisera against T-2 toxin, 50 mCi of ^{3}H-T-2 toxin, 6 mg of DOVE-HS-BSA, 2 mg of DOVE-HS-peroxidase, 2.5 mg DOVE-HS-polylysine and 5 ml of antiserum against STX were prepared and delivered. In addition, 10 mg of T-2-HS-IgG (goat) and 13 mg of DOVE-HS-IgG (goat) were prepared and delivered to Dr. Hunter. Methods for preparation of such deliverables were described either in the present report or in our first annual report (August 15, 1982).

III. DISCUSSION AND ASSESSMENT OF WORK DONE

In the second year of the contract, considerable progress in improving the production of antibody against T-2 toxin in rabbits was made. High titers of antibody were obtained from rabbits after prolonged repeated immunization with T-2 conjugates which contained 10-15 moles of hapten per mole of BSA. The sensitivity of RIA was improved considerably by using the higher titer antibody preparation and also by the use of highly specific radioactive T-2 toxin. However, the effects of protein carriers, animal species and alternate sites for conjugation on antibody production have not been studied. Further experiments will be directed to those areas. Because Dr. Hunter's group has most recently succeeded in obtaining high T-2 antibody titers in an immunomodulation system in mice, selection of best immunogen(s) in the rabbit system in our laboratory should help to improve the antibody production in mice further. During 1983, we have also established a protocol for an indirect ELISA of T-2 toxin. This technique has also been tested by USAMRIID. However, there were some problems in its reproducibility. To overcome such problems, future studies on ELISA of T-2 toxin should be directed to better quality control of reagents, uniformity of ELISA protocol, development of a dipping ELISA system as well as additional collaborative studies.

The effect of chain length between T-2 or DAS and BSA on the immunogenic properties of the conjugates was studied. However, different effects were observed with these two toxins. Whereas T-2 HS-BSA appears to be better than T-2 HG-BSA for antibody production against T-2 toxin, DAS-HG-BSA was found to be better than DAS-HS-BSA. Although this difference might be due to a difference in the orientation of mycotoxins around the protein molecule, the stability of the conjugates may also play a role. If the poor immunogenic property of these conjugates is due to an in vivo hydrolysis of the hapten from the protein molecule, then the rate of hydrolysis of the

hemisuccinates and hemiglutarates may directly affect the efficiency of these conjugates for antibody production. The response of rabbits after each booster injection (Figs. 1 and 2) suggests that there might be some problems in this regard.

In the present study, antibodies against both DAS and DOVE were obtained from rabbits after immunizing with DAS-HG (or HS)-BSA and DOVE-HS-BSA. Although the antibody titers were considerably less than T-2 antibody titers, because we have prepared very highly specific radioactive ^{3}H-DAS and ^{3}H-DOVE, these antibodies were adequate for RIA of these mycotoxins. The DAS antibodies appear to be primarily specific for DAS. Antibodies against DOVE, however, are shown to cross-react with DAS and also T-2 toxin. Thus, they may also be useful for the analysis of other trichothecene mycotoxins. For the analysis of macrocyclic-type trichothecenes, the toxins should be first hydrolyzed to their corresponding alcohols before being subjected to the RIA or ELISA. Because highly specific radioactive ^{3}H-DAS and ^{3}H-DOVE were obtained most recently (July, 1983) in our laboratory, detailed studies to characterize these antibodies as well as to develop ELISAs for these mycotoxins will be carried out.

Difficulties still exist for the production of specific antibodies against vomitoxin. Considerable efforts were made to characterize the antisera by testing the reactivities of different antibodies with different enzyme conjugates during the past year. Currently, we are testing the cross-reactivities of these antibodies with tritiated reduced-VT, DAS, T-2, and DOVE. After completion of these studies, we should be able to understand the nature of such antibodies and to improve the antibody production.

For STX, we have obtained antibodies which are capable of neutralizing the toxic effect as well as being useful in the ELISA. The nature of the cross-linking of the toxin to BSA is poorly understood. Further studies should be directed to characterize the reaction between BSA and STX by reacting STX with some model compounds, to study the cross-reactivities of the antibodies with STX derivatives and analogues with the antibodies, as well as to search for methods of improving the antibody titers. In addition, antibodies obtained from other conjugates should be characterized further.

IV. LITERATURE CITED

A. Publications published:

1. Fontelo, P. A., Beheler, J., Bunner, D. L. and Chu, F. S. (1983) Detection of T-2 toxin by an improved radioimmunoassay. Appl. Environ. Microbiol. 45:640-643.

2. Chu, F. S. (1983) Immunochemical methods for mycotoxin analysis. In Proceedings Int. Symp. Mycotoxins. p. 170-187.

3. Chu, F. S., Fan, Titan S. L. and Li, S. W. (1983) An indirect ELISA for saxitoxin. Abst. of 1983 AOAC Annual Meeting, Washington, D.C.

4. Lee, S. C., Beery, J. T. and Chu, F. S. (1984) Immunoperoxidase localization of T-2 toxin. Toxicol. Appl. Pharmacol. 72:228-235.

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6. Chu, F. S., Liang, M.-Y. and Zhang, G. S. (1984) Production and characterization of antibody against diacetoxyscirpenol. Appl. Environ. Microbiol. 48:777-780.

7. Chu, F. S., Zhang, G. S., William, M. and Jarvis, B. (1984) Production and characterization of antibody against deoxyverrucarol. Appl. Environ. Microbiol. 48:781-784.

B. Manuscripts in preparation:

1. Chu, F. S., Zhang, G. S. and Bischoff, W. Improved conditions for production of antibody against T-2 in rabbits.

2. Zhang, G. S. and Chu, F. S. Preparation and characterization of hemisuccinate and hemiglutarate of T-2 toxin and diacetoxyscirpenol.

Table I. Cross-reactivity of vomitoxin antibodies with free VT and different VT derivatives and conjugates.[a]

Test Ligands	Antibody[b] / Antigens[c]:	CMO-VT-BSA / CMO-VT-Peroxidase	CMO-VET-BSA / CMO-VET-PL	NS-VT-BSA / HS-VT-PL
VT		>10^5	>10^5	>10^5
CMO-VT		>10^5	--	--
HS-VT		10^5	--	10^5
HS-VET[d]		15,700	--	--
TGA-VT		10^5	--	--
CMO-VT-BSA		1,480	10^4	1370
CMO-VT-PL		1,000	1.72 x 10^4	1380
HS-VT-BSA		1,000	1700	1580
HS-VT-PL		1,630	1.35 x 10^4	1500
TGA-VT-BSA		1,120	1000	950
TGA-VT-PL		1,400	--	--
HS-VET-BSA		1,500	1630	1100
HS-VET-PL		--	>10^5	--
CMO-VET-BSA		1,570	>10^5	1570
BSA		>10^5	>10^5	>10^5
PL		>10^5	>10^5	>10^5

[a]Both direct and indirect ELISA were used in the analysis. The numbers in the columns indicate the concentration (ng/ml, 50 µl samples were used in each test) required to inhibit 50% binding of the antibody to the marking antigens.

[b]Antibody that is obtained after immunization with respective conjugates.

[c]Marker antigens coated to the plate.

[d]Abbreviations: most are in the text except: VET, triacetyl VT; TGA, thioglycolic acid; PL, polylysine.

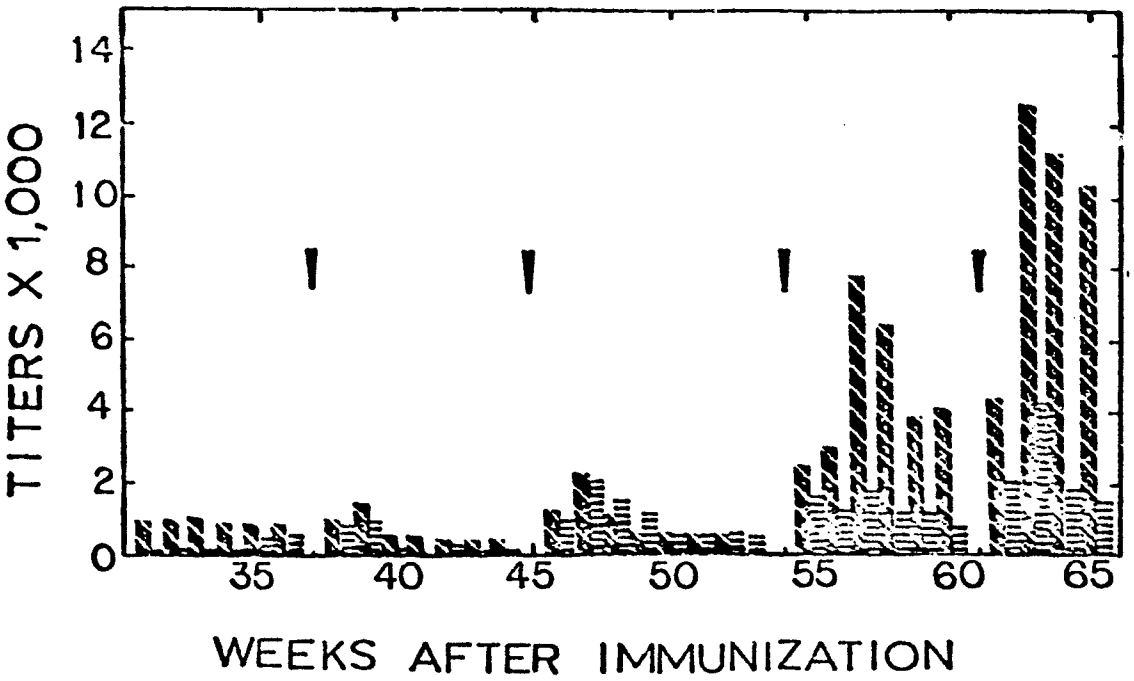

Fig. 1. Antibody titers for two representative rabbits (highest and lowest) after immunizing with different T-2 HS-BSA preparations. Between week 0-20 (at 0, 7 and 12 weeks), the rabbits were immunized with a conjugate which had a molar ratio (T-2 to BSA) of 30. At week 20 and thereafter, preparations with molar ratios of between 12-15 were used for the booster injections. Before 30 weeks, the titers were below 500. All the rabbits received 300-500 μg of conjugates in each injection. The arrows indicate time of booster injections.

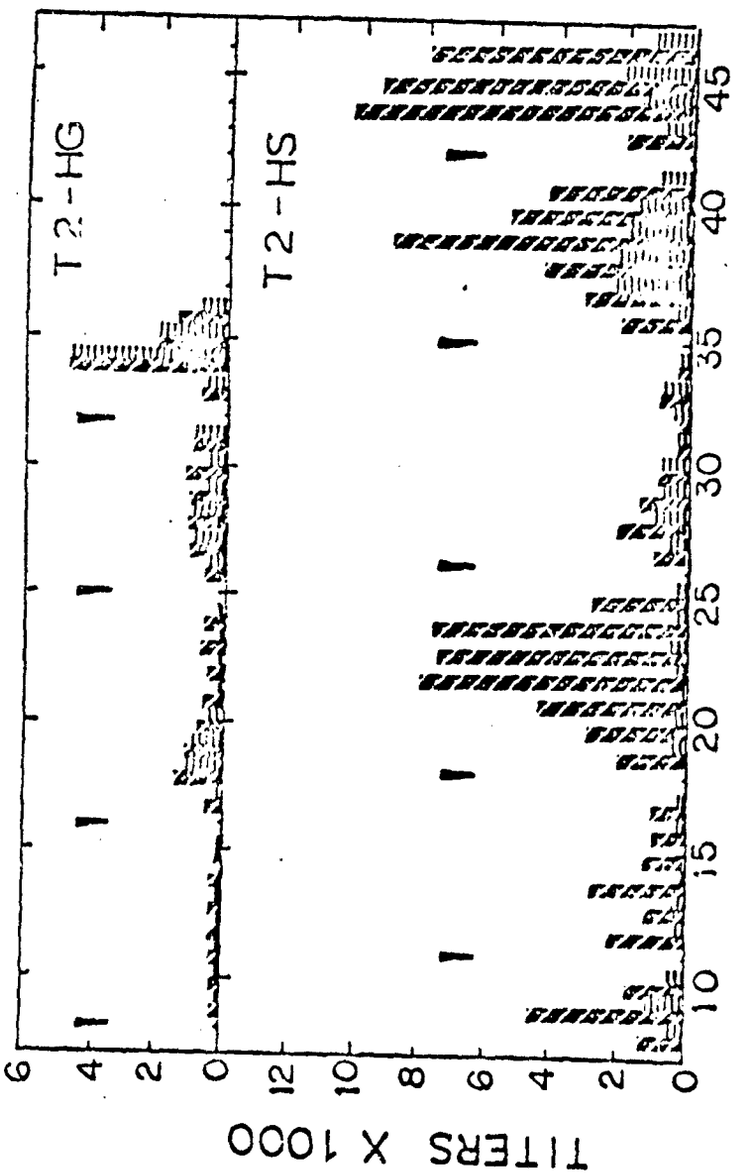

Fig. 2. Antibody titers of representative rabbits after immunizing with BSA conjugates of T-2-HS and T-2-HG with molar ratio of 12-15. All the rabbits received 300-500 µg of conjugate in each injection. The arrows indicate time of booster injections.

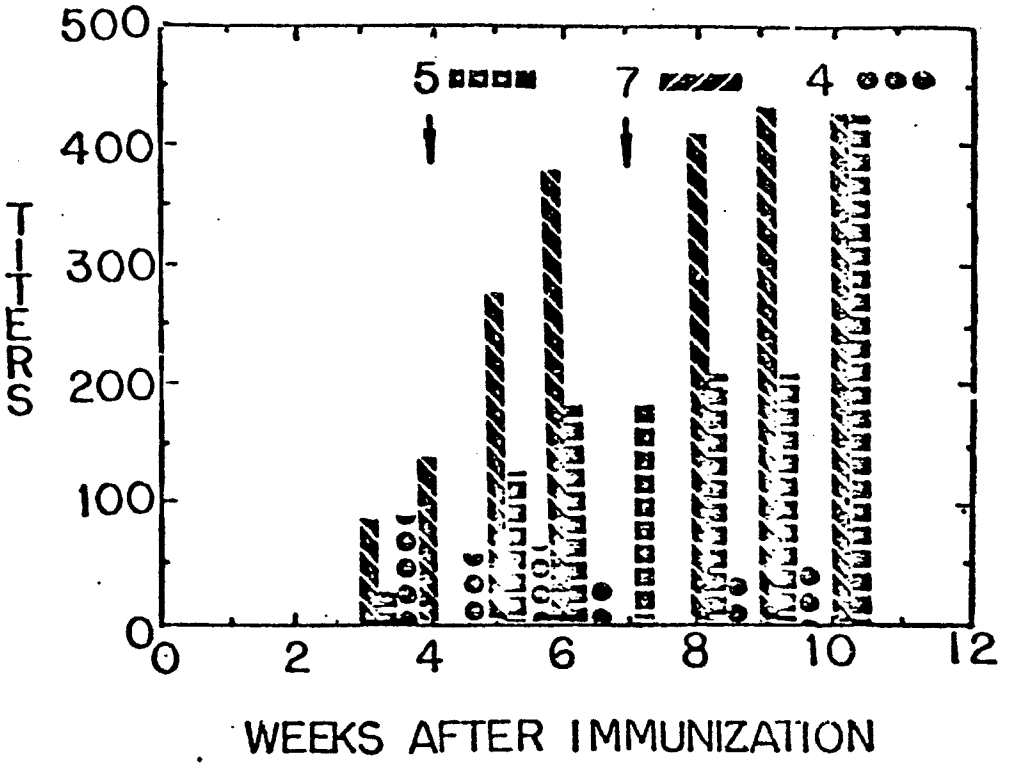

Fig. 3. Antibody titers of representative rabbits after immunizing with BSA conjugates of DAS-HS (No. 4) and DAS-HG (No. 5 & 7). All the rabbits received 300 µg of conjugate in the initial injection and 250 µg for the boosters as indicated by arrows. The immunization time for rabbit No. 4 should be the number showing in the X axis plus 20. The titer is defined as the reciprocal of the amount of antiserum (ml) required to bind 50% of 7,000 cpm of tritiated DAS which had specific radioactivity around 19.5 Ci/mmole.

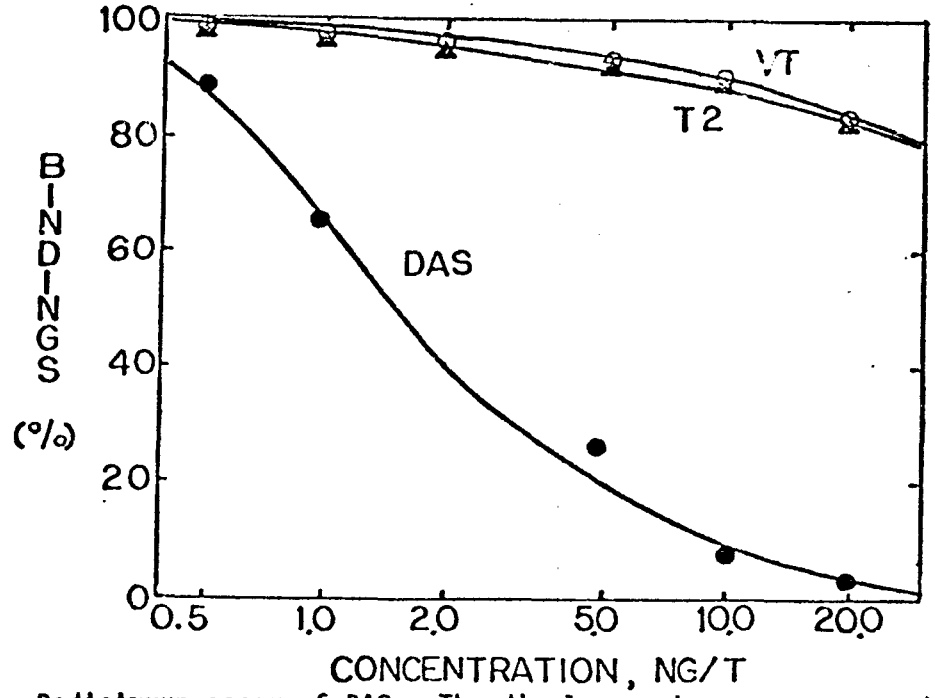

Fig. 4. Radioimmunoassay of DAS. The displacement curves were established from data in an experiment in which a one to 400 dilution of rabbit serum against DAS was incubated with 7,000 cpm of tritiated DAS in the presence of different amounts of unlabelled DAS, VT, T-2, and DOVE. Separation of free and bound was achieved by an ammonium sulfate precipitation method.

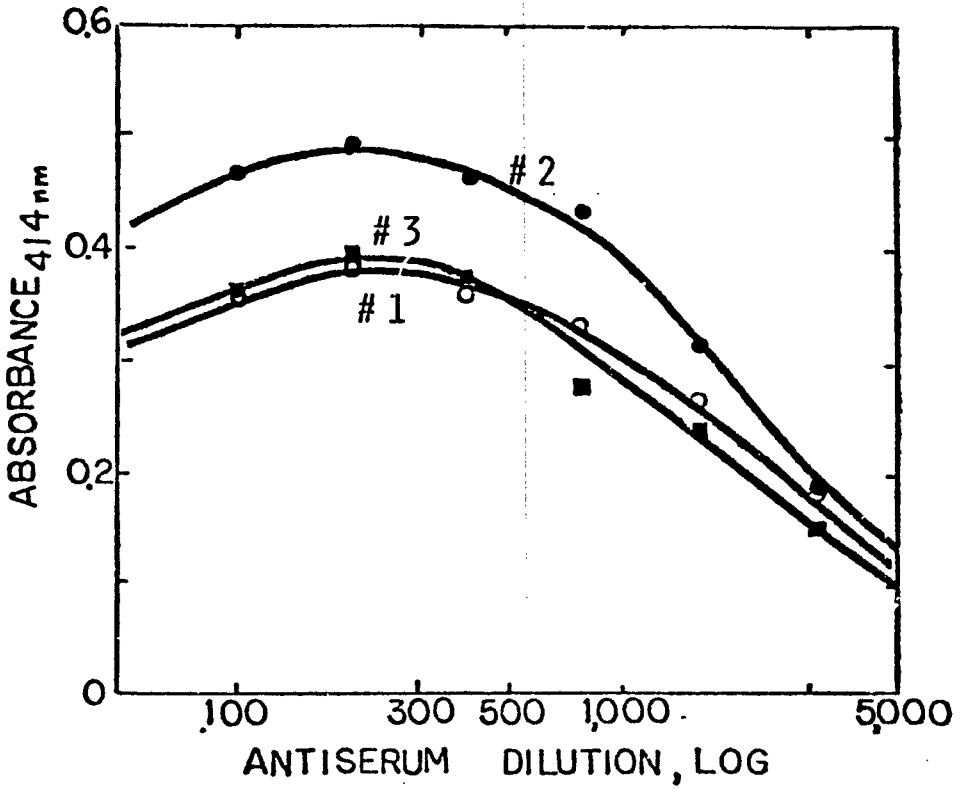

Fig. 5. Determination of antibody titers by direct ELISA for VT. The antibody titers shown in this figure represent 3 rabbits 8 weeks after immunizing with 400 μg of carboxymethyl oxime (CMO) of VT conjugated to ovalbumin (OVA). A booster injection was made at the 7th week. A CMO-VT-peroxidase preparation was used as the marker enzyme.

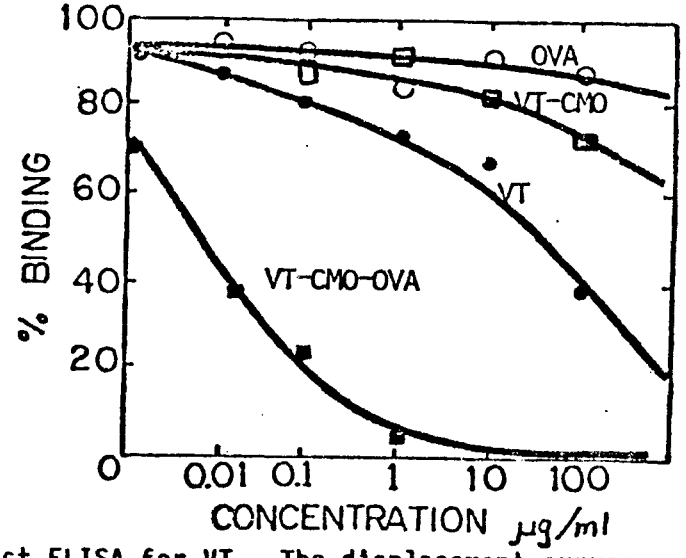

Fig. 6. A direct ELISA for VT. The displacement curves were established from data of a direct ELISA in which antiserum (CMO-VT-OVA, 1 to 200 dilution) and CMO-VT-peroxidase (50 µl of 10 µg/ml solution) were incubated with different testing ligands. The minimal detection level for VT was around 5 ng/assay or 0.1 µg/ml.

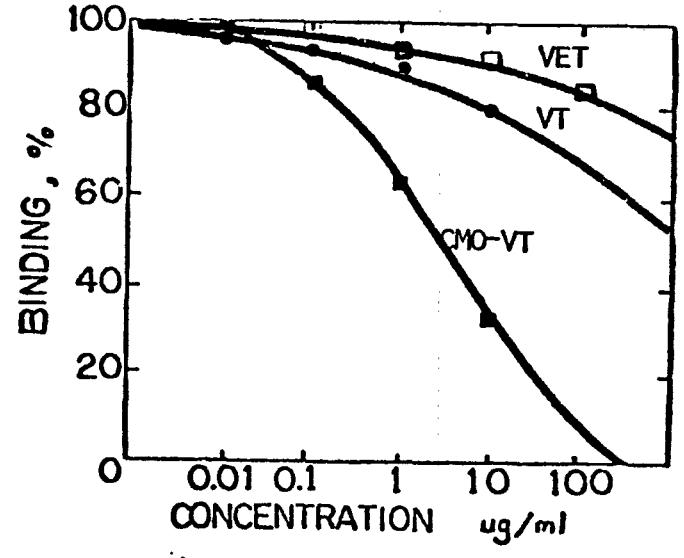

Fig. 7. Indirect ELISA for VT. The displacement curves were established from an experiment in which CMO-VT-polylysine was coated to the plate, and then incubation with rabbit antiserum (1:5,000 dilution) in the presence of different testing ligands, followed by incubation with goat antirabbit-IgG-peroxidase conjugate. The minimal detection level for VT is around 50 ng/assay or 1 µg/ml.

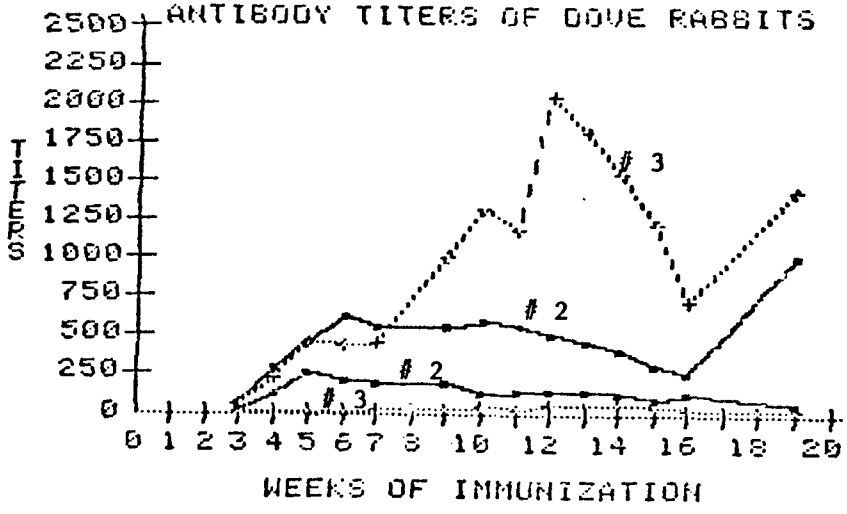

Fig. 8. Antibody titers of rabbits (No. 2 and 3) after immunizing with DOVE-HS-BSA. The experiments were carried out under the same conditions as in Fig. 3 and tritiated DAS (lower 2 curves) and DOVE (top 2 curves) were used as the marker ligands. The definition of titers is also the same as Fig. 3.

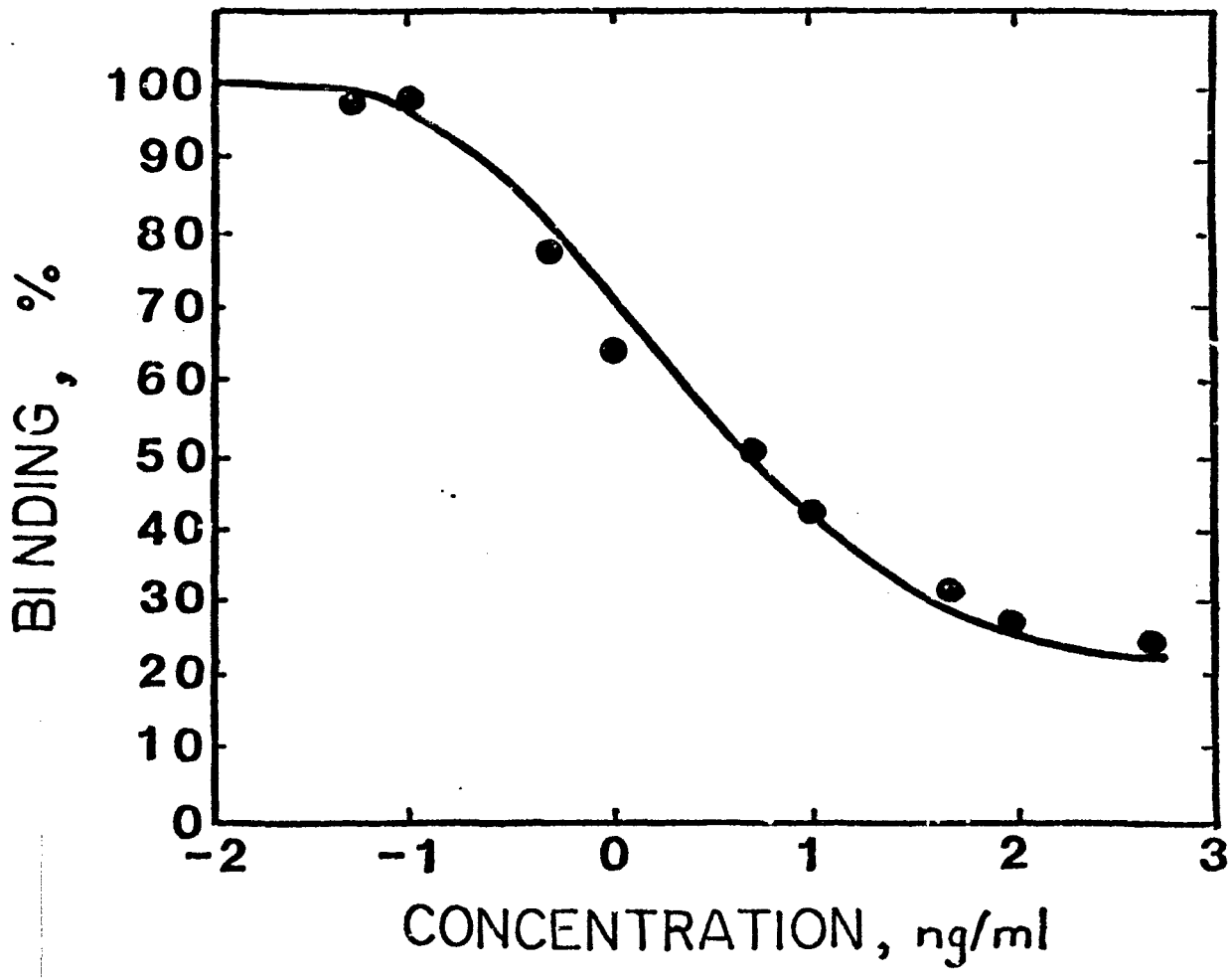

Fig. 9. Indirect ELISA of STX. In this typical experiment, STX-BSA (0.16 µg/well) was coated to the assay plate by the bicarbonate method. STX antibody at a dilution of 1 to 5,000 (0.1 µg/well) was incubated together with various STX. The minimal detection level for STX was around 25 pg in each assay (or 0.5 ng/ml). The X axis is in log scale.

APPENDIX I

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An Indirect Enzyme-Linked Immunosorbent Assay for T-2 Toxin in Biological Fluids

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(Received for publication March 16, 1984)

ABSTRACT

An indirect enzyme-linked immunosorbent assay (ELISA) which can detect 0.2 to 1 ng of T-2 toxin per ml in urine, serum and milk was developed. T-2 hemisuccinate was conjugated to polylysine which was then coated to a microtiter plate and incubated with rabbit anti-T-2 antibody and sample extract. The amount of anti-T-2 antibody bound to the plate was then determined by reaction with goat anti-rabbit IgG-peroxidase complex and by subsequent reaction with the substrate. Samples spiked with T-2 toxin were subjected to a simple cleanup procedure by passing them through a reversed-phase Sep-Pak catridge (C_{18}). The recoveries of tritiated T-2 toxin added to the urine, serum and milk samples were between 71 to 90% after the cleanup step. In the ELISA, significant interference was observed when more than 5 µl of sample, without cleanup treatment, were used in each analysis. After cleanup, extracts equivalent to 50 µl of serum, urine or milk per well did not significantly interfere with the assay. The recoveries of T-2 toxin added to serum (1 to 10 ng/ml), urine (0.2 to 10 ng/ml) and milk (0.2 to 10 ng/ml) after cleanup treatment as determined by the indirect ELISA were found to be 51 to 82%, 73 to 82% and 80 to 83%, respectively.

APPENDIX II

TOXICOLOGY AND APPLIED PHARMACOLOGY 72, 228–235 (1984)

Immunoperoxidase Localization of T-2 Toxin[1]

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Immunoperoxidase Localization of T-2 Toxin. LEE, S. C., BEERY, J. T., AND CHU, F. S. (1984). *Toxicol. Appl. Pharmacol.* 72, 228–235. Antibody against T-2 toxin was used for monitoring the fate of T-2 toxin in mice given a single po dose of 11 mg/kg by the peroxidase–antiperoxidase (PAP) method. T-2 toxin was demonstrable in the esophagus from 5 min to about 24 hr postdosing. In the stomach, T-2 toxin was detected within the cytoplasm of intact and injured epithelial cells. In the duodenum, T-2 toxin was primarily localized within the surface epithelium and phagocytic elements (macrophages and neutrophils) of the duodenal lamina propria, especially toward the tips of the villi. Following sloughing of duodenal villous tips, the recovering villous tip epithelial cells frequently showed both cytoplasmic and nuclear T-2 toxin. The jejunum showed weak T-2 toxin within the cytoplasm of villous tip epithelial cells only. The ileum never demonstrated T-2 toxin. Tissue response in the gastrointestinal (GI) tract was characterized by transient edema, marked cytolysis and sloughing, and a subsequent leukocytic invasion of the stomach and proximal small intestine. Evidence of severe gastric and less severe duodenal bleeding was apparent and associated with a marked loss of gastric epithelium and intestinal villous tips. The kidney medulla contained the majority of T-2 toxin stain. T-2 toxin was noted within the distal tubular cells, the cells of the collecting tubules, and the epithelium covering the papilla. T-2 toxin was never demonstrated in any of the hepatic tissue examined.

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